

## NEUTRALIZATION OF VIRUSES BY HOMOLOGOUS IMMUNE SERUM

### I. QUANTITATIVE STUDIES ON FACTORS WHICH AFFECT THE NEUTRALIZATION REACTION WITH NEWCASTLE DISEASE, INFLUENZA A, AND BACTERIAL VIRUS, T<sub>3</sub>

BY DAVID A. J. TYRRELL, M.R.C.P., AND FRANK L. HORSFALL, JR., M.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

(Received for publication, March 17, 1953)

Under natural conditions, reactions between viruses and antibodies occur in living host tissues. Because of the importance of such reactions in immunity to virus infections, much attention has been directed toward them. The most nearly natural model which is available for detailed study in the laboratory appears to be the reaction in which the infectivity of a virus is neutralized by homologous antibody.

In quantitative studies carried out some years ago (1, 2) on the neutralization of influenza A virus by immune serum in the mouse lung, it was demonstrated that there is a linear relationship between the logarithm of the amount of virus neutralized and the logarithm of the serum dilution end point. The slope of the neutralization line which relates the two variables was found to be 1.4, indicating that the ratio between virus and antibody was not constant and changed progressively, though gradually, as one variable was altered with respect to the other. Almost identical results were obtained in comparable neutralization experiments with Western equine encephalomyelitis virus in the mouse brain (3), pneumonia virus of mice in the mouse lung (4), herpes simplex virus in newborn mice (5), and influenza viruses not adapted to the mouse lung (6). In support of the finding that the slope of the neutralization line is greater than 1.0, it was demonstrated (7) that the infectivity of neutralized influenza A virus can be reactivated on dilution of the mixture when tested in the mouse.

With poliomyelitis virus in tissue culture, it appears that the slope of the neutralization line is approximately 1.0 (8), while, with influenza A virus in suspended chorio-allantoic membrane *in vitro*, there is evidence suggesting that the slope may have a value as high as 3.0 (9).

Neutralization experiments in the chick embryo have given results very different from those obtained with the same agents in the mouse. The slope of the neutralization line with influenza A or B virus and homologous immune serum in the allantoic cavity is remarkably steep and has shown values ranging from 3.0 to 4.7 (10, 11). Under similar experimental conditions, the slopes obtained with Newcastle disease virus (10) or mumps virus (12) appeared to be about as steep as those obtained with

influenza viruses. On the other hand, when different routes of inoculation were employed in the chick embryo, the value of the slope was much lower. Thus, in experiments on the chorioallantoic membrane, the slope of the neutralization line with Newcastle disease virus was found to be about 1.4 (13), while, with herpes simplex virus in the yolk sac, a slope in the neighborhood of 2.0 was shown (14).

In view of the striking differences which have appeared in the results of quantitative studies of the neutralization reaction with viruses and the lack of an adequate explanation for such differences, a further investigation was undertaken. The results of a series of studies concerned with a number of variables which affect the reaction as seen with Newcastle disease, influenza A, or the bacterial virus, T<sub>3</sub>, are the subject of this paper. In the accompanying paper (15), the theoretical aspects of these findings are considered and a quantitative hypothesis based on multiple equilibrium reactions is developed.

#### *Materials and Methods*

*Viruses.*—The following strains of influenza A virus were utilized: PR8, WS, and NWS, a neurotropic variant of the WS strain which was obtained originally by Stuart-Harris (16). In addition, the following strains of Newcastle disease virus (NDV) were employed: Hickman and B, a relatively avirulent variant which was obtained from Dr. F. B. Bang. With each virus strain, 9 or 10 day old chick embryos were inoculated intra-allantoically with 0.2 ml. of a 10<sup>-9</sup> dilution of infected allantoic fluid or mouse lung suspension. The embryos were incubated at 35°C. for 42 to 48 hours and then were chilled quickly and the allantoic fluids harvested. Infected fluids were pooled and stored in 1.0 ml. volumes in nitrocellulose tubes at -65°C. in the absence of gaseous CO<sub>2</sub> (17). Portions of some pools were dialyzed against 0.1 M phosphate buffer, pH 7.2, and stored at 4°C. for use in hemagglutination-inhibition tests. For experiments with the bacterial virus, T<sub>3</sub>, was added to a culture of *Escherichia coli* B multiplying in aerated nutrient broth.<sup>1</sup> After the bacterial culture underwent lysis, it was filtered, and the filtrate was stored at 4°C.

*Immune Sera.*—Immune sera against each of the influenza and Newcastle disease virus strains were prepared in rabbits by intravenous injection of 10 ml. of infected allantoic fluid followed by intraperitoneal injections of 10 ml. of similar fluid at 2 to 3 week intervals. Serum was collected at various periods after the 2nd week of immunization. Convalescent chicken serum against the B strain of Newcastle disease virus was prepared as described by Bang *et al.* (13). Antiserum against bacterial virus T<sub>3</sub> was prepared in rabbits by repeated intravenous injection of infected culture filtrates.<sup>2</sup> Sera were stored at 4°C. without preservative.

*Red Blood Cells.*—Blood was drawn aseptically from the wing veins of normal roosters, mixed with acid citrate dextrose solution (18), pooled, and stored at 4°C. for 1 to 5 days. Before use, the red blood cells were washed 3 times in phosphate buffered saline and 0.5 per cent suspensions in similar saline were prepared.

*Infectivity Titrations.*—Serial dilutions of infected allantoic fluid or virus suspension were made in chilled buffered saline which, when chick embryos were to be inoculated, contained 1000 units of penicillin and 1 mg. of streptomycin per ml. The dilutions were made in steps corresponding to either 1.0 or 0.5 logarithmic unit and were kept in an ice bath until inoculations were completed.

<sup>1</sup> Both T<sub>3</sub> and *E. coli* B were kindly provided by Dr. W. F. Goebel.

<sup>2</sup> The anti-T<sub>3</sub> serum was kindly provided by Dr. G. T. Barry.

For inoculation of chick embryos, one of two routes was used: 0.2 ml. of diluted virus was placed on the dropped chorioallantoic membrane or injected into the allantoic cavity of 9 or 10 day embryonated eggs. Inoculated eggs were incubated at 35°C. In certain experiments, incubation was continued for 42 hours, after which the eggs were quickly chilled. Allantoic fluid was then removed from each egg and 0.1 ml. was mixed with 1.0 ml. of 0.5 per cent chicken RBC suspension. Allantoic fluids which caused hemagglutination were taken as evidence of infection of the embryos. In other experiments, incubation was continued for 6 days. Eggs were candled daily and the embryos which died between the 2nd and 6th day after inoculation were considered to be infected.

For inoculation of mice, one of two routes was used: Groups of 4 to 6 Swiss albino mice, 3 to 4 weeks old, were inoculated intranasally under ether anesthesia; each mouse received 0.05 ml. of diluted virus. Deaths were recorded daily and the survivors were killed after 10 days' observation and the lung lesions scored as described previously (1). Similar groups of mice were inoculated intracerebrally under light ether anesthesia with 0.03 ml. of diluted virus. Those mice which died between the 3rd and 10th day were considered to have been infected.

For surviving tissue suspensions, portions of chorioallantoic membrane were suspended in 1.0 ml. of chemically defined medium in test tubes (inside diameter, 13 mm.). The procedure for the preparation of the medium and membranes was that described by Fulton and Armitage (19). 0.1 ml. of diluted virus was added to each suspended membrane and the tubes were shaken mechanically at 90 strokes per minute for approximately 60 hours at 35°C. The membrane was then removed from the tube and 0.3 ml. of 0.5 per cent chicken RBC suspension was added to the medium. Those tubes which showed hemagglutination were considered to have contained infected membranes. 50 per cent infectivity end points were calculated by the method of Reed and Muench (20).

*Neutralizing Antibody Titrations.*—All sera were heated at 56°C. for 30 minutes before dilution. To serial twofold or 3.16-fold dilutions of serum prepared in buffered saline was added an equal volume of a diluted virus suspension. The diluted serum-virus mixtures were shaken and then were held at room temperature for about 20 minutes or were incubated at 37°C. for 1 hour. They were then transferred to an ice bath where they were maintained until inoculation was completed. The routes of inoculation employed with chick embryos and mice, as well as the procedures for the determination of 50 per cent serum dilution end points were identical with those used in virus titrations. The procedure employed in neutralization experiments with bacterial virus T<sub>2</sub> is described in the experimental section.

*Hemagglutination Titrations.*—These were carried out by the fractional dilution procedure recently developed in this laboratory (21). In brief, this procedure makes use of three series of virus dilutions in which the dilution steps correspond to decrements of 0.1 logarithmic unit. The procedure yields virus titration end points reproducible within  $\pm 10$  per cent.

*Hemagglutination-Inhibiting Antibody Titrations.*—These also were carried out by the fractional dilution procedure (21). The procedure yields antibody titration end points reproducible within  $\pm 8$  per cent.

#### EXPERIMENTAL

*Neutralization Experiments with NDV in Ovo.*—In neutralization experiments with the B strain of Newcastle disease virus (NDV) on the chorioallantoic membrane of the chick embryo, Bang *et al.* (13) found that the slope of the neutralization line, which relates the immune serum dilution end point to the amount of virus neutralized, was only slightly greater than 1.0. In earlier neutralization experiments of a similar kind with influenza or New-

castle disease viruses, other workers (10, 11) had shown that the slope of the neutralization line determined in the allantoic cavity was very steep and had a value of about 4.0. The difference in the slope recently reported (13) for NDV neutralization and that obtained previously with influenza viruses and

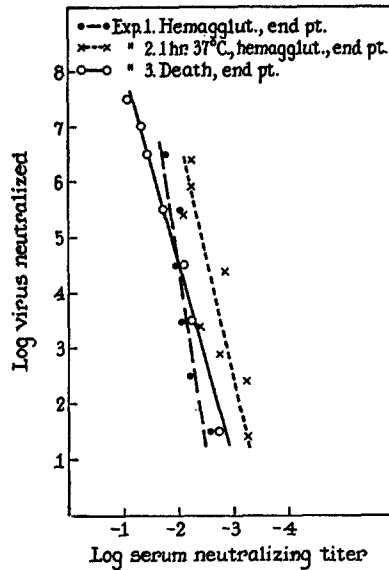


FIG. 1

FIG. 1. Results of neutralization experiments with NDV (Hickman strain) and one immune rabbit serum in the allantoic cavity of the chick embryo. Dilutions of both virus and serum were prepared in steps equivalent to 0.5 log unit. Log virus neutralized = log virus titer (determined under identical conditions) — log virus dilution used. An analysis of the results is given in Table I.

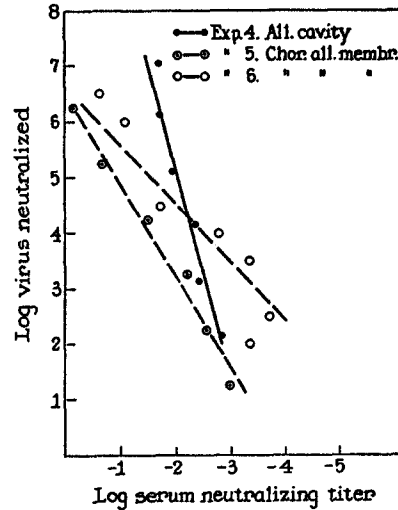


FIG. 2

FIG. 2. Results of neutralization experiments with NDV (Hickman or B strain, Experiment 6) and homologous immune sera (rabbit or convalescent chicken, Experiment 6) in the allantoic cavity or on the chorioallantoic membrane of the chick embryo. Dilutions of both virus and serum were prepared in steps equivalent to 0.5 log unit. An analysis of the results is given in Table I.

NDV in the same host species was so marked that an explanation for the discrepancy was sought.

In the recent neutralization experiments with NDV (13), chicken immune serum-virus mixtures were incubated for 1 hour at 37°C. and then were placed on the dropped chorioallantoic membrane. Death of 50 per cent of the embryos within 6 days was taken as the titration end point. In the earlier experiments with influenza viruses and NDV (10, 11), rabbit immune serum-virus mixtures were held at 4°C. and then were injected into the al-

lantoic cavity. A positive hemagglutination reaction with 50 per cent of allantoic fluids obtained after 48 hours was taken as the titration end point. To determine what effect these differences in experimental procedure had upon the slope of the neutralization line, each variable was studied separately with NDV and homologous immune sera.

The results of experiments concerned with individual variables in the neutralization procedure are presented in Figs. 1 and 2. An analysis of the results and the slope of the neutralization lines obtained are given in Table I. The details of the techniques employed are described above.

TABLE I  
*Neutralization Experiments in Embryonated Eggs with Newcastle Disease Virus and Homologous Immune Sera*

Experiment No.	NDV strain	Anti-serum*	Incubation of serum-virus mixture at 37°C.	Route of inoculation	Criterion for 50 per cent infectivity end point	Slope of neutralization line	Standard deviation of slope
			<i>hrs.</i>				
1	Hickman	R6	0	In allantoic cavity	HA†	5.8	0.85
2	"	"	1	" " "	"	3.3	0.42
3	"	"	0	" " "	Death§	3.6	0.50
4	"	R3	0	" " "	HA	4.1	0.47
5	"	"	0	On chorioallantoic membrane	Death	1.6	0.12
6	B	Ch7	1	" " "	"	1.2	0.17

\* R, immune rabbit serum; Ch, convalescent chicken serum.

† HA, hemagglutination after 2 days' incubation.

§ Death of embryos 2 to 6 days after inoculation.

As shown in Fig. 1, when neutralization experiments with NDV were performed in the allantoic cavity, the slope of the neutralization lines was very steep and ranged from 3.3 to 5.8 (*cf.* Table I). The slope was but little affected by incubation of serum-virus mixtures at 37°C. for 1 hour or by the titration end point used. End points determined from death of embryos within 6 days or hemagglutination of allantoic fluids at 2 days yielded similar slopes. The slope of the lines shown in Fig. 1 corresponds well with that found previously under similar experimental conditions with influenza viruses (11) and is similar to that reported for NDV neutralization in the allantoic cavity (10).

It should be noted that, on incubation of serum-virus mixtures at 37°C., considerably more virus was neutralized by a given serum dilution as is indicated by the shift of the neutralization line to the right. Results of a similar sort have been secured previously with NDV (13), influenza (11), and herpes simplex viruses (14).

As illustrated in Fig. 2, when neutralization experiments with NDV were carried out on the chorioallantoic membrane, the slope of the neutralization lines was not steep and ranged from 1.2 to 1.6 (*cf.* Table I). The experimental procedure used with the B strain of NDV and convalescent chicken serum was identical with that described by Bang *et al.* (13), and the results obtained closely confirm those previously reported by these workers. It should be emphasized that experiments with exactly the same virus strain and immune serum gave, in the allantoic cavity, a slope of 4.1 (*cf.* Experiment 4, Table I) and, on the chorioallantoic membrane, a slope of 1.6 (*cf.* Experiment 5, Table I). When both immune serum and virus dilutions were prepared in normal allantoic fluid, and the mixtures were inoculated on the chorioallantoic membrane, the slope of the neutralization line with NDV was not steeper than that obtained with the same materials diluted in buffered saline.

Regression lines were fitted to the experimental data by the method of least squares. The lines, so obtained, are shown in Figs. 1 and 2 together with the experimental points. The regression coefficients and their standard deviations are given in Table I.

The values given for the standard deviations of the slopes were computed from the estimate of the variance of each slope thus obtained. It is clear that the only significant differences in the values of the slopes shown in Table I are those between neutralization experiments carried out in the allantoic cavity and those performed on the dropped chorioallantoic membrane. The other variables studied, *i.e.* type of immune serum, incubation of serum-virus mixtures, and criterion for titration end point, did not cause a significant effect upon the slope of the neutralization line.

It appears clear that the chief factor which affected the slope of the neutralization line with NDV was the route by which the serum-virus mixtures approached susceptible cells in the chorioallantoic membrane. When the mixture is placed on the dropped chorioallantoic membrane, it spreads out into a thin layer on a relatively dry surface and comes in contact with ectodermal cells forming the external layer of the membrane. On the other hand, when the mixture is injected into the allantoic cavity, it is considerably diluted by the allantoic fluid and comes in contact with endodermal cells forming the internal layer of the membrane. These differences in the host cell-virus system may be important factors which contribute to the marked differences found in neutralization slopes with NDV in the chick embryo.

*Neutralization Experiments with PR8 in Ovo.*—Because the route of inoculation was the chief factor which affected the slope of the neutralization line in experiments with NDV, it appeared probable that this variable might also be important in similar experiments with influenza virus in the chick embryo.

The results of neutralization experiments with the PR8 strain of influenza A virus are shown in Fig. 3. An analysis of the results and the slope of the

neutralization lines, along with the standard deviations, computed as described above, are shown in Table II. It is clear that the slope obtained on neutralization in the allantoic cavity, *i.e.* 2.5, is considerably steeper than that found in a comparable experiment on the dropped chorioallantoic membrane, *i.e.*, 1.6. In addition, it was found that in neutralization experiments carried

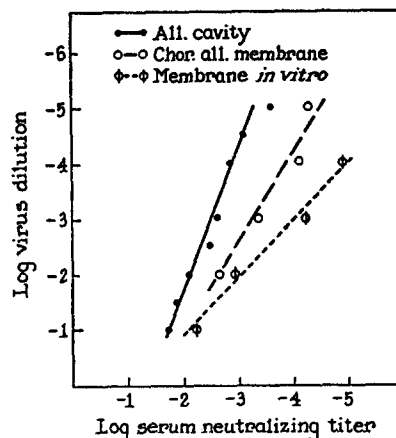


FIG. 3. Results of neutralization experiments with PR8 and one immune rabbit serum in the allantoic cavity, on the chorioallantoic membrane, or in chorioallantoic membrane suspension *in vitro*. Log virus dilution used is given on the vertical axis. An analysis of the results is given in Table II.

TABLE II  
*Neutralization Experiments with Influenza A Virus (PR8 Strain) and Homologous Immune Serum*

Neutralization carried out:*	Slope of neutralization line†	Standard deviation of slope
In allantoic cavity	2.5	0.80
On chorioallantoic membrane	1.6	0.30
In chorioallantoic membrane suspension <i>in vitro</i>	1.0	0.12

\* The PR8 strain and one immune rabbit serum were used in all experiments.

† Criterion for infectivity end point was hemagglutination.

out in surviving membranes suspended *in vitro*, as described above, the slope has a value of 1.0.

These results serve to support those obtained with NDV and tend to affirm the idea that the route of inoculation in the chick embryo is a critical factor in determining the slope of neutralization lines.

*Neutralization Experiments with NWS in Ovo and in Mice.*—To enlarge on this evidence, the NWS strain of influenza A virus afforded a number of ad-

vantages. With this strain of virus, it is feasible to carry out neutralization experiments in either the chick embryo or the mouse, as well as in surviving membrane suspensions *in vitro*, and to employ two different routes of inoculation in each animal species. Thus, the slope of the neutralization line obtained with one virus strain and one immune serum under five distinct sets of experimental conditions can be compared.

The NWS strain was rendered fully pathogenic for the mouse on either intracerebral or intranasal inoculation by rapid serial passage. In all experiments one pool of allantoic fluid

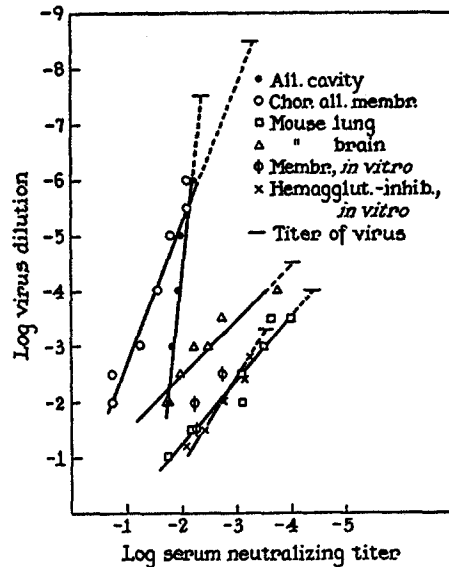


FIG. 4. Results of neutralization experiments with NWS (single pool of infected allantoic fluid) and one immune rabbit serum in five host-cell systems. Log virus dilution used is given on the vertical axis. The titer of the virus found in each host-cell system is shown. An analysis of the results is given in Table III.

obtained from eggs inoculated with a  $10^{-2}$  dilution of infected mouse lung suspension was employed. In each experiment, virus dilutions were prepared from a fresh aliquot of the frozen allantoic fluid pool. Serum dilutions were prepared freshly from one specimen of serum secured from a rabbit immunized with NWS. Although the various neutralization experiments were done successively, the materials and procedures used were identical. Only the routes of inoculation and the host species were varied.

In Fig. 4, the results of five different types of neutralization experiment with the NWS strain and a single immune serum are presented. Each type of experiment was carried out on at least two separate occasions and the results were found to be closely reproducible. An analysis of the results along with the slope of the neutralization lines and the standard deviations, computed as described above, are shown in Table III.

With NWS, much the steepest slope was found in neutralization experi-



ments in the allantoic cavity. This was the case also with NDV and PR8. The value of the slope corresponds with those values found previously in comparable experiments with influenza viruses (10, 11). When the mixtures were inoculated on the chorioallantoic membrane, the slope of the neutralization line was considered less steep, as was found also with NDV and PR8. However, with NWS, the value of the slope obtained by this route of inoculation was somewhat greater than with NDV or PR8.

In contrast to the findings in the chick embryo, the slope obtained on intracerebral or intranasal inoculation in the mouse, as well as with surviving membrane suspensions *in vitro*, approximated a value of 1.0. These findings are in good agreement with those reported earlier with other viruses in the

TABLE III  
*Neutralization Experiments with Influenza A Virus (NWS Strain) and Homologous Immune Serum in Various Host-Cell Systems*

Neutralization carried out:*	Titer of virus in host tissue used	Neutralizing titer of serum†	Slope of neutralization line	Standard deviation of slope
	<i>-log</i>	<i>-log</i>		
On chorioallantoic membrane	8.5	0.9	2.6	0.17
In allantoic cavity	7.5	1.8	4.7	1.20
In mouse brain	4.5	2.1	1.0	0.12
In mouse lung	4.0	3.1	1.2	0.15
In chorioallantoic membrane suspension <i>in vitro</i>	3.7	2.8	—	—
Hemagglutination-inhibition <i>in vitro</i>	3.3	3.0	1.3	0.10

\* A single pool of allantoic fluid infected with the NWS strain of influenza A virus and one immune rabbit serum (R1) were used in all experiments.

† Determined in each case with infected allantoic fluid diluted  $10^{-2.5}$ .

mouse lung (1, 2, 4) or the mouse brain (3). In membrane suspensions with NWS, there were too few experimental points to establish the slope, but relatively high serum titers were obtained with several virus dilutions.

It will be observed from the data shown in Fig. 4 that the neutralizing titer of the serum, determined against a constant dilution of virus, was dependent upon the host-cell system used in neutralization experiments. When the various neutralizing titers obtained with a virus dilution of  $10^{-2.5}$ , as shown in Table III, are compared with the virus titers found in the same host-cell systems, an inverse relationship appears. Inoculation by either route in chick embryos gave high virus titers and on neutralization yielded relatively low serum titers. Conversely, in the mouse lung and with membrane suspensions *in vitro*, low virus titers were found but on neutralization relatively high serum titers were obtained. A comparison between these data and others secured with bacterial virus T<sub>3</sub> is presented below.

*Neutralization Experiments with WS in Ovo.*—To determine the effect of

one further variable on the slope of the neutralization line, experiments were carried out with the WS strain of influenza A virus. Two specimens of immune serum from one rabbit were used. One specimen was obtained about 3 weeks and the other 9 weeks after beginning immunization with WS.

As is shown in Table IV, the ratios between the neutralizing and the hemagglutination-inhibiting titers of the two serum specimens were distinctly different. This was to be expected on the basis of earlier studies (11). Despite this difference in the antibodies present, the two serum specimens yielded, in neutralization experiments in the allantoic cavity, slopes which had closely similar values. Thus, it appears that late rabbit immune serum gives, in the

TABLE IV  
*Neutralization Experiments with Influenza A Virus (WS Strain) and Early or Late Homologous Immune Serum*

Serum*	Antibody titer†			Slope of neutralization line‡		Standard deviation of slope
	Hemagglutination-inhibiting	Neutralizing	Ratio Neutralizing/Hemagglutination-inhibiting	No. experimental points		
<i>days</i>						
20	8192	740	0.09	9	4.3	0.33
63	3072	1000	0.33	9	3.6	0.50

\* Early and late immune sera were obtained from one rabbit (R2).

† Expressed as the reciprocal.

‡ Neutralization was carried out in the allantoic cavity.

allantoic cavity, a neutralization slope about as steep as that found with early immune serum.

*Reactivation of Neutralized WS on Dilution.*—When the slope of a neutralization line is greater than 1.0, reactivation of neutralized virus should be obtained on dilution of the mixture *in vitro* if the reaction is reversible. The steeper is the slope of the line, the more readily should reactivation be demonstrable for dilution decreases the concentrations of components in a mixture equally, *i.e.*, in accord with a slope of 1.0. On this basis, it appeared that reactivation ought to be most easily obtained in experiments in which inoculation into the allantoic sac was employed.

The WS strain of influenza A virus and homologous immune rabbit serum were used. Two series of serum-virus mixtures were prepared: In one, serial dilutions of both materials were made separately, mixed, and held at room temperature for 20 minutes. In the other, a mixture containing equal volumes of a serum dilution of 1:10 and a virus dilution of  $10^{-3.0}$  was made and then diluted serially. The final dilutions, which corresponded exactly to those in the first series, were held at room temperature for 40 minutes.

On testing the infectivity of the two series of mixtures in the allantoic cavity, the results shown in Fig. 5 were obtained. It is clear that reactivation of neutralized WS occurred on dilution and that infective virus reappeared when the mixture was diluted sufficiently. As was to be expected, infective virus became demonstrable first when the dilutions of the components in the neutral mixture corresponded to those which did not cause neutralization when separately diluted materials were mixed. However, not all the neutralized virus reappeared; only about 10 per cent of that present in the original mixture became reactivated on dilution. It will be noted that, when very high dilutions of serum and virus were mixed, there was no definite effect upon the

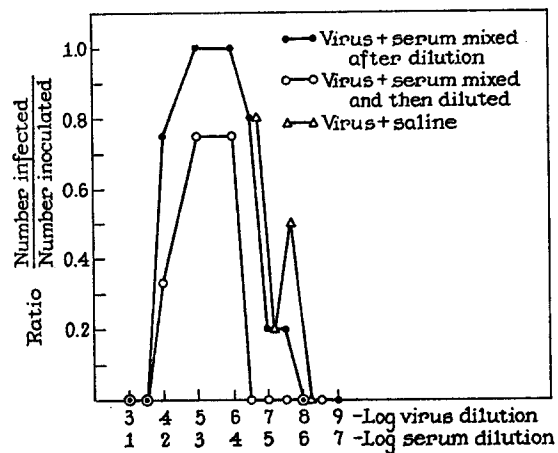


FIG. 5. Results of dilution-reativation experiments with WS and homologous immune serum in allantoic cavity of the chick embryo.

infectivity end point as compared with the control. These results serve both to confirm and to extend those obtained previously by Taylor (7) and Burnet (10) with neutralized influenza virus mixtures in the mouse lung and in the chick embryo, respectively.

*Neutralization Experiments with  $T_3$ .*—Bacterial virus  $T_3$  and homologous immune serum were employed in additional neutralization experiments for two reasons: Firstly, to determine whether the linear relationship between the logarithm of the serum titer and the logarithm of the virus dilution employed held with this different system. Secondly, to determine whether the precise quantitation possible with a bacterial virus system would shed light on some of the problems raised in the experiments with animal viruses described above.

The experimental procedure was closely comparable to that used in neutralization experiments with animal viruses and differed in a number of features from previously reported neu-

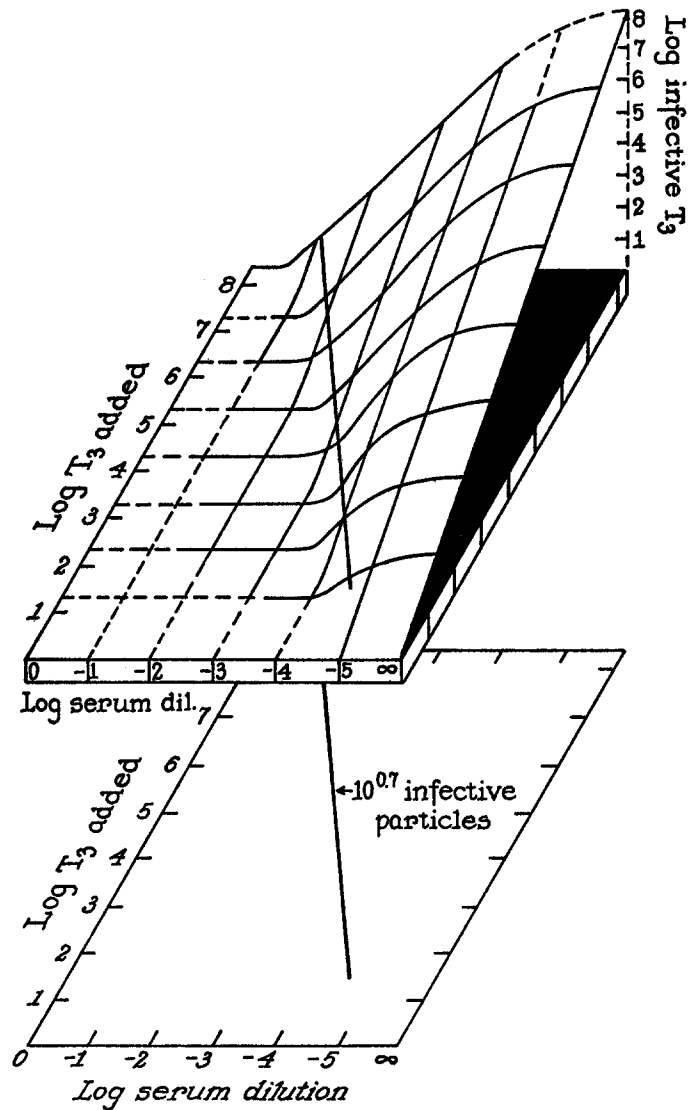


FIG. 6. Graphic representation in three dimensions of the results of a complete neutralization experiment with  $T_3$  and one immune rabbit serum. The curves shown in the upper portion of the graph were drawn on the basis of 42 experimental points. The solid lines in the curves show the range of the three variables used. The broken lines were drawn as extensions of the solid lines to complete the surface of the graph. The straight line in the upper portion of the graph intersects each curve at a point indicating that five  $T_3$  particles remained infective. This line was projected onto the lower portion of the graph which has the conventional two dimensional form.

tralization experiments with bacterial viruses. Dilutions of  $T_3$  were prepared in nutrient broth and mixed with an equal volume of immune serum dilutions. The mixtures were held at 37°C. for 90 minutes so as to obtain nearly maximal neutralization of the virus. The amount of unneutralized  $T_3$  was then determined as follows: When only small quantities of unneutralized  $T_3$  were expected, 0.2 ml. of the mixture was added to warm nutrient agar containing *E. coli* B and plated for subsequent plaque count. When large quantities of unneutralized  $T_3$  were expected, 0.2 ml. of the mixture was added to 1.8 ml. of cold nutrient broth containing about  $10^8$  *E. coli* B per ml. This mixture was shaken thoroughly and then centrifuged at 11,000 g for 10 minutes in a cold centrifuge. The supernatant was discarded and the bacteria with adsorbed  $T_3$  were resuspended in cold broth. Serial dilutions were prepared in cold broth and plated in the usual manner for plaque counts. This procedure was used to avoid errors due to dilution reactivation of neutralized  $T_3$ .

In neutralization experiments of this type with  $T_3$ , there are three variables: (a) the number of virus particles added to the mixture; (b) the dilution of immune serum in the mixture; and (c) the number of virus particles which remain infective, *i.e.* unneutralized, after incubation of the mixture. The relationships between these three variables, as determined in experiments with  $T_3$  and homologous immune serum, are shown graphically in three dimensional form (22) in Fig. 6. It is clear that, as the dilution of serum was increased, progressively less virus was neutralized and that, as more virus was added, progressively more remained unneutralized.

By means of the series of curves shown in Fig. 6, it is possible to determine the serum dilutions and the virus concentrations which yield a given number of unneutralized virus particles. When this is done and the logarithms of the two variables are plotted, as in Fig. 7, there emerge neutralization lines which closely resemble those obtained with the various animal viruses studied. It is evident that, when the titration end point is taken as  $10^{0.7}$ ,  $10^{8.0}$ , or  $10^{5.0}$  unneutralized  $T_3$  particles, there is, in each case, a linear relationship between the logarithms of the serum dilution and the quantity of virus added. Moreover, the three neutralization lines shown have slopes which are almost identical with a value of approximately 2.0.

These neutralization lines can be described by the empirical relationship:—

$$\log V = a \cdot \log A + \log v + k \quad (1)$$

in which  $V$  is the quantity of virus added,  $A$  is the serum concentration,  $v$  is the quantity of unneutralized virus,  $a$  is the slope, and  $k$  is a constant. This equation is closely similar to that originally used (1, 2) to describe the neutralization of influenza A virus in the mouse lung. Moreover, the equation implies that the so called percentage law, previously proposed (23) as a description of the neutralization of bacterial viruses, is valid because  $\log (V/v) = a \cdot \log A + k$ .

From the results shown in Fig. 7, it is evident that, as more and more unneutralized  $T_3$  particles were taken to represent the titration end point, the neutralizing titer of the serum, determined against a constant quantity of

virus, progressively increased. It will be recalled that an analogous relationship was found in experiments described above with NWS in various host-cell systems. As shown in Table III, as more and more virus was needed to give an infectivity end point with NWS in the host-cell systems utilized, the serum neutralizing titer, determined against a  $10^{-2.5}$  dilution of virus, progressively

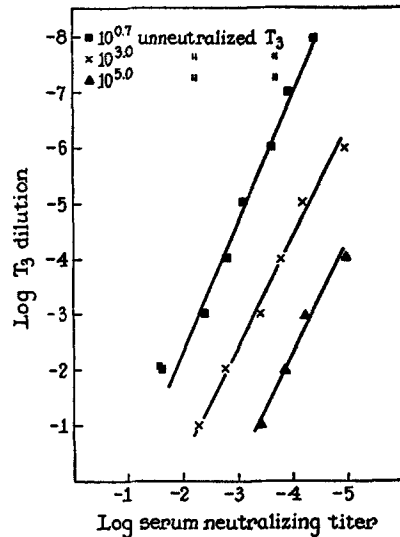


FIG. 7

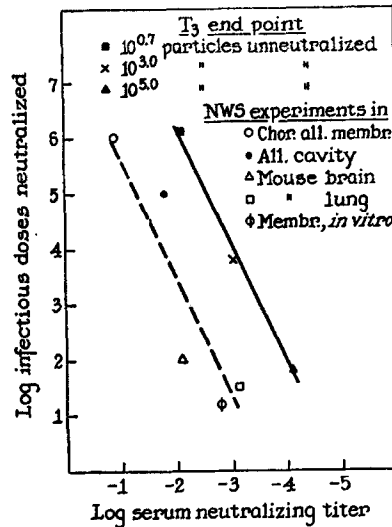


FIG. 8

FIG. 7. Relationship between the neutralizing titer of one immune rabbit serum against  $T_3$  and the number of unneutralized  $T_3$  particles chosen to represent the titration end point.

FIG. 8. Relationship between the neutralizing titer of immune serum against NWS or  $T_3$  and the number of infectious doses of virus neutralized when the amount of virus added to the mixture was constant. With  $T_3$ , an infectious dose was defined as equal to the number of unneutralized particles chosen for the titration end point. Log infectious doses neutralized = log virus titer (determined in the host-cell system used with NWS or in arbitrary units with  $T_3$ ) — log virus dilution (*i.e.*  $-2.5$ ) employed. In all cases the neutralizing titer of the serum was that obtained with a constant dilution (*i.e.*  $10^{-2.5}$ ) of either NWS or  $T_3$ .

increased. The similarity in the two relationships is shown in Fig. 8 in which the logarithm of the number of infectious doses of virus neutralized is plotted against the logarithm of the serum dilution end point. In the three titrations with  $T_3$  (*cf.* Fig. 7) and in the five with NWS (*cf.* Fig. 4), in all of which a virus dilution of  $10^{-2.5}$  was used, a similar relationship appears evident. The slope of the line for  $T_3$  has a value of 2.1 with a standard deviation of 0.45. It should be emphasized that, had a different virus dilution been selected for analysis of the titrations with NWS, a line with a different slope would have emerged.

The correlation between the results obtained with  $T_3$  and NWS suggests a

reasonable explanation for the effect of the susceptibility of the host-cell system on the neutralizing titer of immune serum. In the more resistant host-cell systems, *e.g.* mouse lung and membrane suspensions *in vitro*, a relatively large amount of unneutralized virus is needed to cause demonstrable infection. Under these circumstances, less antibody is required because fewer virus particles need to be neutralized. Conversely, in the more susceptible host-cell systems, *e.g.* chorioallantoic membrane and allantoic cavity, a relatively small amount of unneutralized virus is sufficient to cause infection. Therefore, more antibody is required because more virus particles require neutralization.

#### DISCUSSION

The results obtained in this investigation show that the quantitative relationship between virus and immune serum in neutralization experiments is affected markedly by certain variables in the procedure utilized. The most important variable appears to be the host-cell system used and, in the case of the chick embryo, the route of inoculation has a striking effect.

It should be emphasized that even when the most carefully controlled techniques are employed, estimates of the slope of neutralization lines are subject to a moderate error. The steeper is the slope, the larger will be the error in the values assigned to the slope. Numerous replicate experiments are required to establish the value of a slope with reasonable precision (1). The data presented in this paper, although based on numerous experimental points, do not warrant the fitting of any but linear regression equations.

The results of neutralization experiments with the bacterial virus,  $T_3$ , are compatible with those obtained by earlier workers (23-26). Moreover, they provide confirmation of the validity of the so called percentage law and amplify previous evidence by demonstrating the relationship between the proportion of virus neutralized and the concentration of immune serum. In addition, they show that, although the number of unneutralized  $T_3$  particles chosen as end point affects the position, or intercept, of the neutralization line, this variable does not affect the slope of the line.

The evidence indicates that, in experiments with the animal viruses utilized, differences in the position of the neutralization line may be explained in a similar manner. Marked differences in the amount of virus required to cause infection in the different host-cell systems studied are correlated with changes in the position of the line. However, as with  $T_3$ , the slope of the neutralization line is not dependent upon this variable.

These findings bear upon the choice of a technique for neutralization experiments with animal viruses. If the concentration of a large amount of antibody is to be measured, a host-cell system highly susceptible to infection with the virus can be used satisfactorily (11). If, on the other hand, the objective is to detect small amounts of antibody, it is important to use only a small number of infective doses of virus as is evident from the linear relationship between the

two variables. However, it is still more important to employ a host-cell system which is relatively insusceptible and develops an infection only when a large amount of unneutralized virus is present. As an example, the neutralizing titer of an immune serum against NWS was 20-fold higher when determined in the mouse lung than when measured in the allantoic cavity of the chick embryo even though 10 infective doses of the agent was used in each species (*cf.* Fig. 4). However, 10 infective doses in the mouse represents 7,000-fold more virus than does 10 infective doses in the chick embryo.

Neutralization experiments may be carried out by either the constant virus-varying serum or the constant serum-varying virus technique. In the present study, both techniques were employed, in effect, for each component was varied relative to the other. If the slope of the neutralization line with a given system is greater than 1.0, and the constant serum-varying virus procedure is used, a small difference in antibody concentration causes a larger change in virus titer. Thus, in a system with a slope of 3.0, a tenfold increase in antibody concentration causes a 1,000-fold decrease in virus titer. In addition, only a small range of antibody concentration is measurable unless a virus preparation with an extremely high titer is used.

The constant virus-varying serum technique has the advantage that it is unaffected by the slope of the neutralization line and reflects differences in antibody concentration directly. With but few exceptions, the slope of neutralization lines is greater than 1.0. The steeper is the slope of the line, the greater is the precision of this procedure because large differences in the amount of virus used cause only small changes in the titer of the serum (11). In addition, the range of serum dilutions which can be employed is unlimited and a virus preparation with a low titer can be used. In general, the constant virus-varying serum technique is more likely to yield satisfactory results in a system in which the slope is not known.

It is evident from the results of these studies that a mixture of serum and virus which appears to be completely neutralized, as judged by tests in one host-cell system, may be highly infective in another. Because of the marked differences in the position and the slope of the neutralization lines, many mixtures of influenza virus and immune serum which are non-infective in the mouse are infective in the chick embryo (*cf.* Fig. 4). This is dependent largely upon the fact that the amount of unneutralized virus required to infect the mouse is much greater than that required to infect the chick embryo. Similar findings were reported previously with vaccinia virus (27) or equine encephalomyelitis viruses (28) in mixtures with homologous immune serum. These are probably to be explained in the same manner.

#### SUMMARY

Neutralization experiments with Newcastle disease, influenza A, or bacterial virus, T<sub>3</sub>, reveal, under all conditions studied, a linear relationship between



the logarithm of the serum dilution end point and that of the quantity of virus used. With Newcastle disease and influenza A, the slope of the neutralization line varies markedly with the host-cell system used and in the chick embryo is strikingly affected by the route of inoculation. The other variables examined have no definite effect upon the slope. Reactivation of neutralized influenza virus is demonstrable in the chick embryo on dilution of the mixture.

There appears to be an inverse relationship between the degree of susceptibility of a host to infection with influenza A virus and the neutralizing titer of a serum as measured in that host. With the T<sub>3</sub>-serum system, comparable results are obtained when the number of unneutralized virus particles chosen as the end point is varied widely.

## BIBLIOGRAPHY

1. Horsfall, F. L., Jr., *J. Exp. Med.*, 1939, **70**, 209.
2. Horsfall, F. L., Jr., and Lennette, E. H., *J. Exp. Med.*, 1941, **73**, 327.
3. Morgan, I. M., *J. Immunol.*, 1945, **50**, 359.
4. Horsfall, F. L., Jr., and Curnen, E. C., *J. Exp. Med.*, 1946, **83**, 25.
5. Kilbourne, E. D., and Horsfall, F. L., Jr., *J. Immunol.*, 1951, **67**, 321.
6. Kilbourne, E. D., and Horsfall, F. L., Jr., *J. Immunol.*, 1951, **67**, 431.
7. Taylor, R. M., *J. Immunol.*, 1941, **40**, 373.
8. Ledinko, N., Riordan, J. T., and Melnick, J. L., *Am. J. Hyg.*, 1952, **55**, 323.
9. Fulton, F., *J. Hyg.*, 1952, **50**, 265.
10. Burnet, F. M., *Australian J. Exp. Biol. and Med. Sc.*, 1943, **31**, 231.
11. Walker, D. L., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1950, **91**, 65.
12. Henle, W., *et al.*, *J. Immunol.*, in press.
13. Bang, F. B., Foard, M., and Karzon, D. T., *Bull. Johns Hopkins Hosp.*, 1950, **87**, 130.
14. Jawetz, E., and Coleman, V. R., *J. Immunol.*, 1952, **68**, 645.
15. Tyrrell, D. A. J., *J. Exp. Med.*, 1953, **97**, 863.
16. Stuart-Harris, C. H., *Lancet*, 1939, **1**, 497.
17. Horsfall, F. L., Jr., and Ginsberg, H. S., *J. Bact.*, 1951, **61**, 443.
18. Rapoport, S., *J. Clin. Inv.*, 1947, **26**, 591.
19. Fulton, F., and Armitage, P., *J. Hyg.*, 1951, **49**, 247.
20. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.
21. Horsfall, F. L., Jr., and Tamm, I., *J. Immunol.*, 1953, **70**, 253.
22. Hopkins, E. F., *Science*, 1947, **106**, 326.
23. Andrewes, C. H., and Elford, W. J., *Brit. J. Exp. Path.*, 1933, **14**, 367.
24. Burnet, F. M., Keogh, E. V., and Lush, D., *Australian J. Exp. Biol. and Med. Sc.*, 1937, **15**, 227.
25. Hershey, A. D., Kalmanson, G., and Bronfenbrenner, J., *J. Immunol.*, 1943, **46**, 281.
26. Delbrück, M., *J. Bact.*, 1945, **50**, 137.
27. Andrewes, C. H., *J. Path. and Bact.*, 1928, **31**, 671.
28. Olitsky, P. K., and Harford, C. G., *J. Exp. Med.*, 1938, **68**, 173.